Original Paper

Down-Regulation of the Na⁺-Coupled Phosphate Transporter NaPi-IIa by AMP-Activated Protein Kinase

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Key Words

Energy depletion • Phosphate transport • Compound C • Kidney

Abstract

Background/Aims: The Na⁺-coupled phosphate transporter NaPi-IIa is the main carrier accomplishing renal tubular phosphate reabsorption. It is driven by the electrochemical Na⁺ gradient across the apical cell membrane, which is maintained by Na⁺ extrusion across the basolateral cell membrane through the Na⁺/K⁺ ATPase. The operation of NaPi-IIa thus requires energy in order to avoid cellular Na⁺ accumulation and K⁺ loss with eventual decrease of cell membrane potential, Cl⁻ entry and cell swelling. Upon energy depletion, early inhibition of Na⁺-coupled transport processes may delay cell swelling and thus foster cell survival. Energy depletion is sensed by the AMP-activated protein kinase (AMPK), a serine/threonine kinase stimulating several cellular mechanisms increasing energy production and limiting energy utilization. The present study explored whether AMPK influences the activity of NaPi-IIa. **Methods:** cRNA encoding NaPi-IIa was injected into *Xenopus* oocytes with or without additional expression of wild-type AMPK (AMPK^{α1}-HA+AMPK^{β1}-Flag+AMPK^{γ1}-HA), of inactive AMPK^{αK45R} (AMPK^{α1K45R}+AMPK^{β1}-Flag+AMPK^{γ1}-HA) or of constitutively active AMPK^{γR70Q} (AMPK^{α1}-HA+AMPK^{β1}-Flag+AMPK^{γR70Q}). NaPi-IIa activity was estimated from phosphate-induced current in dual electrode voltage clamp experiments. **Results:** In NaPi-IIa-expressing, but not in water-injected *Xenopus* oocytes, the addition of phosphate (1 mM) to the extracellular bath solution generated a current (I_p), which was significantly decreased by coexpression of wild-type AMPK and of AMPK^{γR70Q} but not of

AMPK α K45R. The phosphate-induced current in NaPi-IIa- and AMPK-expressing *Xenopus* oocytes was significantly increased by AMPK inhibitor Compound C (20 μ M). Kinetic analysis revealed that AMPK significantly decreased the maximal transport rate. **Conclusion:** The AMP-activated protein kinase AMPK is a powerful regulator of NaPi-IIa and thus of renal tubular phosphate transport.

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Introduction

The AMP-activated protein kinase (AMPK) senses the cytosolic AMP/ATP concentration ratio and thus the energy status of the cell [1, 2]. When activated by energy deficiency, AMPK stimulates several cellular functions serving ATP generation [3], such as cellular glucose uptake, glycolysis, fatty acid oxidation and up-regulation of enzymes required for ATP production [2, 4-6]. AMPK further inhibits energy-utilizing mechanisms including protein synthesis, gluconeogenesis and lipogenesis [2-4]. The kinase thus protects against cell death during energy depletion [3, 7, 8].

In renal proximal tubules, energy is mainly utilized by transepithelial transport [9]. Na⁺-coupled transport processes are driven by the electrochemical gradient for Na⁺, which is maintained by the energy-consuming Na⁺/K⁺ ATPase in the basolateral cell membrane [10]. Energy depletion is expected to impair the activity of the Na⁺/K⁺ ATPase with eventual dissipation of the Na⁺ and K⁺ gradients, depolarization of the cell membrane, cellular accumulation of Cl⁻, osmotic water entry and thus cell swelling [11]. Na⁺-coupled carriers include the Na⁺-coupled phosphate transporter NaPi-IIa (SLC34A1), the most important carrier accomplishing renal tubular phosphate transport across the apical brush border membrane of proximal renal tubules [12-14]. Renal tubular phosphate reabsorption is regulated by a wide variety of parameters, such as dietary phosphate intake, acid-base status, parathyroid hormone, 1,25-(OH)₂ vitamin D₃, FGF-23, insulin and insulin-like growth factor IGF1 [15-21]. Signaling involved in the regulation of NaPi-IIa includes the protein kinases A and C, ERK1/2, Klotho and the PI3K/PKB/GSK3 kinase cascade [22-29]. Nothing is known, however, about the potential regulation of NaPi-IIa by AMPK. The present study thus explored whether AMPK regulates NaPi-IIa.

Materials and Methods

Constructs

For generation of cRNA [30], constructs were used encoding wild-type NaPi-IIa [31], wild-type AMPK (AMPK α 1-HA+AMPK β 1-Flag+AMPK γ 1-HA) [32, 33], constitutively-active AMPK γ R70Q-HA (AMPK α 1-HA + AMPK β 1-Flag + AMPK γ R70Q) [34] and kinase dead mutant AMPK α K45R-HA (AMPK α 1K45R + AMPK β 1-Flag + AMPK γ 1-HA) [35]. The cRNA encoding wild type NaPi-IIa or encoding AMPK was synthesized as described previously [36].

Voltage clamp in *Xenopus* oocytes

Dissection of *Xenopus laevis* ovaries, collection and handling of the oocytes have been described elsewhere [37]. Oocytes were injected with 15 ng of cRNA encoding NaPi-IIa on the first day with or without 4.6 ng of cRNA encoding either AMPK α 1-HA+AMPK β 1-Flag+AMPK γ 1-HA (wild-type AMPK), or AMPK α 1-HA + AMPK β 1-Flag + AMPK γ R70Q (constitutively-active AMPK γ R70Q) or AMPK α K45R-HA + AMPK β 1-Flag + AMPK γ 1-HA (kinase-dead mutant AMPK α K45R) on the second day after preparation of *Xenopus laevis* oocytes [38]. For control, the oocytes were injected with the respective volumes of DEPC-treated water. Therefore, the number of injections and the injected total volume were equal in all oocytes. The electrophysiological experiments were performed at room temperature 3-4 days after the second injection. Where indicated, oocytes were incubated with AMPK activator AICAR (1 mM, Tocris Bioscience, Bristol, UK) or AMPK inhibitor compound C (20 μ M, Calbiochem, Bad Soden, Germany) for the last 24 hours before the measurement. Oocytes were maintained at 17°C in ND96-A solution

containing 88.5 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, 0.11 mM tetracycline (Sigma, Schnellendorf, Germany), 4 μM ciprofloxacin (Fresenius Kabi, Graz, Austria), 0.22 mM refobacin (Merck-Serono, Darmstadt, Germany), 0.5 mM theophylline (Takeda, Singen, Germany) as well as 5 mM sodium pyruvate. The pH was adjusted to 7.4 by adding NaOH. Two electrode voltage-clamp recordings were performed at a holding potential of -50 mV. The flow rate of the superfusion was 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s [39, 40]. The data were filtered at 10 Hz and recorded with a Digidata 1322A A/D-D/A converter and Clampex V.4.02 software for data acquisition and analysis (Axon Instruments, Union City, CA, USA). The data were analyzed with Clampfit V. 9.0.1.07 software (Axon Instruments).

Detection of NaPi-IIa cell surface expression by chemiluminescence

Oocytes were incubated for 20 min in ND96 with 1% BSA at 4°C to block nonspecific antibody binding. Then, oocytes were incubated with primary rabbit anti-human SLC34A1 (NaPi-IIa) polyclonal antibody (diluted 1:500, Life Span Biosciences, WA, USA) for 1 h at 4°C and subsequently with secondary, HRP-conjugated goat anti-rabbit IgG antibody (1:1000, Cell Signaling Technology, Frankfurt, Germany). Oocytes were washed 3 times for 5 min in 1% BSA/ND96 and then 3 times in ND96 without BSA for 5 min at 4°C. Individual oocytes were placed in 96 well plates with 20 μl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) by integrating the signal over a period of 1 s. Results display normalized relative light units. Integrity of the measured oocytes was assessed by visual control after the measurement to avoid unspecific light signals from the cytosol.

Statistical analysis

Data are provided as means $\pm$ SEM, n represents the number of oocytes investigated. All experiments were repeated with at least 3 batches of oocytes. In all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA, and results with $P < 0.05$ were considered statistically significant.

Results

In order to test, whether AMP-activated protein kinase (AMPK) regulates the electrogenic phosphate transporter NaPi-IIa, *Xenopus* oocytes were injected with cRNA encoding NaPi-IIa with or without additional injection of cRNA encoding wild-type AMPK. The electrogenic phosphate transport was minimal in water-injected *Xenopus* oocytes (Fig. 1). In *Xenopus* oocytes expressing NaPi-IIa, however, phosphate (1 mM) induced an inward current (I_p) reflecting electrogenic entry of Na^+ and phosphate. I_p was significantly decreased by additional coexpression of wild-type AMPK ($\text{AMPK}^{\alpha1}\text{-HA} + \text{AMPK}^{\beta1}\text{-Flag} + \text{AMPK}^{\gamma1}\text{-HA}$; Fig. 1). Accordingly, wild-type AMPK inhibited NaPi-IIa activity.

To further characterize the AMPK effect on the phosphate-induced current in *Xenopus* oocytes, we performed kinetic analysis (Fig. 2). In *Xenopus* oocytes expressing NaPi-IIa alone, the maximal current approached 12.2 ± 0.6 nA ($n = 7$) and the phosphate concentration needed for halfmaximal current (K_M) 33.9 ± 11.2 μM ($n = 7$). In *Xenopus* oocytes expressing NaPi-IIa together with wild-type AMPK ($\text{AMPK}^{\alpha1}\text{-HA} + \text{AMPK}^{\beta1}\text{-Flag} + \text{AMPK}^{\gamma1}\text{-HA}$) the maximal transport rate approached 5.5 ± 0.5 nA ($n = 7$) and the phosphate concentration needed for halfmaximal current (K_M) 73 ± 40 μM ($n = 7$). Accordingly, coexpression of AMPK decreased the maximal transport rate without significantly modifying K_M .

Further experiments explored whether the effect of wild type AMPK on NaPi-IIa requires kinase activity. To this end, NaPi-IIa was expressed in *Xenopus* oocytes with or without additional expression of constitutively-active $\text{AMPK}^{\gamma\text{R70Q}}$ ($\text{AMPK}^{\alpha1}\text{-HA} + \text{AMPK}^{\beta1}\text{-Flag} + \text{AMPK}^{\gamma\text{R70Q}}$) or of the catalytically-inactive mutant $\text{AMPK}^{\alpha\text{K45R}}$ ($\text{AMPK}^{\alpha1\text{K45R}} + \text{AMPK}^{\beta1}\text{-Flag} + \text{AMPK}^{\gamma1}\text{-HA}$). As shown in Fig. 3, the electrogenic phosphate transport in NaPi-IIa-expressing *Xenopus* oocytes was markedly decreased by the coexpression of constitutively active $\text{AMPK}^{\gamma\text{R70Q}}$. In contrast, coexpression of catalytically-inactive $\text{AMPK}^{\alpha\text{K45R}}$ did not significantly modify I_p in NaPi-IIa expressing *Xenopus* oocytes.

Fig. 1. Coexpression of AMPK downregulated electrogenic phosphate transport in NaPi-IIa-expressing *Xenopus* oocytes. A: Representative original tracings showing the phosphate (1 mM)-induced current (I_p) in *Xenopus* oocytes injected with water (1) or expressing NaPi-IIa without (2) or with (3) additional coexpression of wild type AMPK. Following removal of extracellular phosphate, the current gradually returned to basal levels. B: Arithmetic means $\pm$ SEM of the normalized phosphate (1 mM)-induced current (I_p) in *Xenopus* oocytes

injected with water (dotted bar, $n = 3$) or expressing NaPi-IIa without (white bar, $n = 11$) or with (black bar, $n = 11$) additional coexpression of wild type AMPK^{wt}. *** indicates statistically significant difference ($p < 0.001$) from current in *Xenopus* oocytes expressing NaPi-IIa alone.

Fig. 2. Wild-type AMPK decreased the maximal current in NaPi-IIa-expressing *Xenopus* oocytes without significantly affecting affinity. Arithmetic means $\pm$ SEM of phosphate-induced current (I_p) as a function of phosphate concentration in *Xenopus* oocytes expressing NaPi-IIa without (white circles, $n = 7$) or with additional coexpression of AMPK^{wt} (black circles, $n = 7$). *, ** indicate statistically significant difference ($p < 0.05$, $p < 0.01$) from current in *Xenopus* oocytes expressing NaPi-IIa alone.

In order to investigate whether the AMPK influences NaPi-IIa surface expression, we used chemiluminescence for determination of membrane NaPi-IIa protein abundance. As

Fig. 3. Constitutively active AMPK^{Y70Q} but not inactive AMPK^{K45R} decreased the current in NaPi-IIa-expressing *Xenopus* oocytes. A: Representative original tracings of phosphate-induced current (I_p) in *Xenopus* oocytes injected with water (1), expressing NaPi-IIa alone (2) or expressing NaPi-IIa with additional coexpression of wild-type AMPK (3), kinase-dead mutant AMPK^{K45R} (4) or constitutively active AMPK^{Y70Q} (5). B: Arithmetic means $\pm$ SEM ($n=17-34$) of the normalized phosphate-induced current (I_p) in *Xenopus* oocytes injected with water (dotted bar, $n=17$), expressing NaPi-IIa alone (white bar, $n=34$) or expressing NaPi-IIa with additional coexpression of wild-type AMPK (1st black bar, $n=24$), of kinase-dead mutant AMPK^{K45R} (2nd black bar, $n=19$) or of constitutively active AMPK^{Y70Q} (3rd black bar, $n=24$). ***($p<0.001$) indicates statistically significant difference from *Xenopus* oocytes expressing NaPi-IIa alone, ##($p<0.01$) indicates statistically significant difference from *Xenopus* oocytes expressing NaPi-IIa and wild-type AMPK.

Fig. 4. Constitutively active AMPK^{Y70Q} but not inactive AMPK^{K45R} decreased NaPi-IIa membrane expression in *Xenopus* oocytes. Arithmetic means $\pm$ SEM ($n=60-82$) of the normalized intensity of the NaPi-IIa-dependent chemiluminescence in *Xenopus* oocytes injected with water (dotted bar, $n=60$), expressing NaPi-IIa alone (white bar, $n=82$) or expressing NaPi-IIa with additional coexpression of constitutively active AMPK^{Y70Q} (1st black bar, $n=75$) or of kinase-dead mutant AMPK^{K45R} (2nd black bar, $n=66$). **($p<0.01$) indicates statistically significant difference from *Xenopus* oocytes expressing NaPi-IIa alone, #($p<0.05$) indicates statistically significant difference from *Xenopus* oocytes expressing NaPi-IIa and wild-type AMPK.

shown in Fig. 4, constitutively active AMPK^{Y70Q} significantly decreased NaPi-IIa cell membrane abundance, an effect not mimicked by catalytically-inactive AMPK^{K45R}.

Finally, we tested whether pharmacological manipulation of AMPK activity similarly influences NaPi-IIa activity. As shown in Fig. 5, a 24 hours exposure of NaPi-IIa-expressing *Xenopus* oocytes to AMPK activator AICAR mimicked the effect of AMPK coexpression. Furthermore, AMPK inhibitor Compound C abrogated the stimulatory effect of AMPK coexpression on NaPi-IIa activity (Fig. 5).

Fig. 5. Pharmacological activation of AMPK decreased the current in NaPi-IIa-expressing *Xenopus* oocytes. A: Representative original tracings showing currents in *Xenopus* oocytes injected with water (1), or expressing NaPi-IIa alone (2) or expressing NaPi-IIa with additional coexpression of wild-type AMPK and incubated for 24 hours without (3), or with (4) 20 μ M Compound C or expressing NaPi-IIa alone and incubated for 24 hours with 1 mM AICAR (5). B: Arithmetic means $\pm$ SEM ($n=10-20$) of the normalized phosphate-induced current (I_p) in *Xenopus* oocytes injected with water (dotted bar, $n=10$), or expressing NaPi-IIa alone (white bar, $n=19$) or expressing NaPi-IIa with additional coexpression of wild-type AMPK (black bars) and incubated for the last 24 hours before measurement without (left black bar) or with (right black bar, $n=10$) 20 μ M AMPK inhibitor Compound C, or expressing NaPi-IIa alone and incubated for the last 24 hours before measurement with AMPK activator AICAR (1 mM; grey bar, $n=15$). *** ($p<0.001$) indicates statistically significant difference from *Xenopus* oocytes expressing NaPi-IIa alone, ## ($p<0.01$) indicates statistically significant difference from *Xenopus* oocytes expressing NaPi-IIa and wild-type AMPK (AMPK^{wt}-HA+AMPK^{R1}-Flag+AMPK^{R1}-HA).

Discussion

The present study reveals a novel regulator of NaPi-IIa, i.e. the AMP-activated protein kinase AMPK. The kinase decreased the electrogenic phosphate transport mediated by NaPi-IIa. The inhibition of NaPi-IIa during energy depletion lowers the Na⁺ uptake across the apical membrane of proximal renal tubules and thus limits the requirement of Na⁺ extrusion by the energy-consuming Na⁺/K⁺ ATPase. Moreover, inhibition of NaPi-IIa decreases the cytosolic phosphate concentration. The decrease of Na⁺ and phosphate entry and the prevention of Na⁺ and Cl⁻ accumulation counteract cell swelling, a consequence of cellular energy depletion [11]. Accordingly, the AMPK-dependent inhibition of NaPi-IIa contributes to the protective effect of AMPK during energy depletion.

The regulation by AMPK adds to the complexity of NaPi-IIa regulation. NaPi-IIa is regulated by a variety of kinases including protein kinase A [41-48], protein kinase B (PKB)/Akt [22, 27], protein kinase C [43, 45, 46], protein kinase G [46], mitogen activated protein (MAP) kinases [23], extracellular receptor kinase (ERK)-1/2 [23] mammalian target of rapamycin mTOR [22, 27], glycogen synthase kinase GSK3 [27], Janus kinase JAK2 [49],

oxidative stress response kinase OSR [38], and STE20/SPS1-related proline/alanine-rich kinase SPAK [50]. NaPi-IIa trafficking is dependent on vacuolar H⁺ ATPase [51] and stability of NaPi-IIa is further regulated by Klotho [26].

In contrast to phosphate transport, AMPK stimulates glucose uptake [2, 4], an effect due to activation of both, the facilitative glucose carriers GLUT1, GLUT2, GLUT3 and GLUT4 [52-63] and the Na⁺-coupled glucose transporter SGLT1 [64]. The uptake of glucose provides the cell with metabolic fuel. AMPK further stimulates glycolysis, fatty acid oxidation and expression of enzymes required for ATP production [2, 4] and thus counteracts ATP depletion.

Energy generation by glycolysis imposes a proton load on the cell by dissociation of lactic acid, and cytosolic acidification inhibits the rate-limiting enzymes of glycolysis [65]. Accordingly, glycolysis can only be maintained, as long as the generated H⁺ ions are extruded. As a matter of fact AMPK has been shown to activate the Na⁺/H⁺ exchanger [66].

AMPK is not only stimulated by increase in the AMP/ATP ratio, but as well by Ca²⁺ [1], by decrease of O₂ partial pressure [67] and by increase of nitric oxide level [68]. Accordingly, alterations of cytosolic Ca²⁺ activity, O₂ supply, and nitric oxide abundance may, at least in theory, modify NaPi-IIa activity and thus renal tubular phosphate transport.

Conclusion

The AMP-activated kinase AMPK decreases the activity of the Na⁺-coupled phosphate transporter NaPi-IIa and thus participates in the regulation of renal tubular phosphate transport.

Conflict of Interests

The authors state that there are no conflicts to declare.

Acknowledgments

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